THE ISOLATION OF COTTON MEMBRANES USING PROTOPLASTS

By

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USING PROTOPLASTS

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CHAPTER I

INTRODUCTION

The disease, Bacterial Blight of Cotton, is caused by the bacterium, Xanthomonas malvacearum (E. F. Sm.) Dow. The bacterium can enter the plant under conditions of high moisture such as in a driving rainstorm or when irrigation is used. Once inside the plant, the bacteria destroy the cells of the plant and the photosynthetic capability of the plant is reduced. This results in a poorer yield of cotton fiber from the plant. Plant breeders have developed resistant varieties, but the yields of cotton fiber from these plants are lower than from the susceptible varieties.

Since two varieties of cotton plants, one resistant (Im-216) and one susceptible (Ac-44) to cotton blight were available, it was decided to isolate protoplasts from the two varieties of plants, isolate membranes from them and compare the differences between the two varieties.

The isolation of protoplasts has been accomplished with many species of plants. Removal of the cell wall allows the investigator to observe the interactions between the host cells and the pathogens in some diseases. The interaction of a fungal toxin with sugar cane protoplasts has been observed. When an interaction between the bacterium or a product of the bacterium with the protoplasts has been observed, the protoplasts could be lysed and their membranes isolated. A comparison of the membranes from the two varieties might give us an insight into

the mechanism of resistance. Protoplasts from leaf or suspension cells could also be used in somatic hybridization experiments to develop new resistant varieties of cotton.

CHAPTER II

LITERATURE REVIEW

A. Protoplasts

Protoplasts of plant cells consist of ordinary cells which have had their cell walls removed. The first isolation of numerous protoplasts was accomplished by tearing up plasmolyzed leaf tissue under a microscope (1). This method was used as recently as 1957 to prepare protoplasts from onions (2). In 1960 Cocking reported the first use of enzyme mixtures containing predominantly cellulase to remove cell walls (3). In 1963 he demonstrated that for some tissues, mixtures containing predominantly pectinases work best for protoplast isolation (4). Takebe, et al. (5) used pectinase to isolate single cells from tobacco leaves, and the isolated cells were then placed in a medium containing cellulase to form protoplasts. Power, et al. (6) modified this procedure and used the pectinase and the cellulase in the same medium. Kassanis and White in 1974 isolated protoplasts in the dark (7).

The main use of protoplasts has been for somatic cell hybridization (8). Protoplasts from two species of tobacco, N. glauca and N. langsdorffi, were isolated and mixed in a 1:1 ratio in a medium containing 0.25M $NaNO_3$ for thirty minutes to stimulate fusion. The protoplasts were collected by low speed centrifugation and plated on a medium on which only the hybrid fused protoplasts would grow. Approximately 0.01% of the fused protoplasts divided and formed callus. The callus formed

rudimentary shoots and leaves in culture but failed to form roots. The regenerated shoots were grafted onto the freshly cut stem surface of young plants of N. glauca. The grafted shoots produced flowers and fertile seed capsules which were found to be identical with the N. glauca X N. langsdorffi sexually produced amphiploid. Kao, et al. (9) have accomplished the fusion of protoplasts from several different species, but they were unable to reproduce the work of Carlson, et al. (8).

The literature contains two reports of the use of protoplasts for the isolation of cell organelles. Moore, et al. (10) reported the use of protoplasts in an attempt to isolate mitochondria from suspension cells. They reported the isolation of intact mitochondria, but the yield was too small for the method to be of any practical use. App, et al. reported the isolation of nuclei from rice cells in culture (11). They reported yields of thirty to forty percent and suggest the method as a good one for the investigation of nuclear processes.

B. Plant Cell Culture

The literature contains few references dealing specifically with cotton callus and suspension cells. Schenk and Hildebrandt (12) first reported the successful culture of cotton callus and suspension cells in 1969. Davis, et al. (13) erroneously reported the 'first' initiation and maintenance of cotton callus cultures in 1974.

Monographs edited by White (14) and Kruse and Patterson (15) as well as a series of publications on the growth in culture of plant cells by Street, et al. (16-18) should be consulted by the interested reader for a more detailed review of plant cell culture.

C. Plant Cell Membranes

The majority of the work isolating plasma membranes from plants has been done on root tissue. Hall in 1969 located the Ca^{++} ATPase of maize roots in electron micrographs by staining the root cells with lead phosphate (19). The ATPase was found chiefly at the outer cell membrane and in regions of the plasmodesmata passing through the cell wall. Hall, et al. (20) found ATPase activity in a cell wall fraction from barley roots. Schultz, et al. found an enrichment of 5' nucleotidase in membrane fractions isolated from Acanthamoeba sp., a photosynthetic amoeba (21). 5' nucleotidase activity was detected in a purified cell wall fraction from Phaseolus vulgaris cotyledons (22). Lai, et al. (23) observed changes in 5' nucleotidase and glucose-6-phosphatase activity in Phaseolus vulgaris cotyledon tissue during germination. A membrane fraction has been isolated from Phaseolus vulgaris cotyledons which shows an enrichment in (Na^{+}-K^{+} ATPase) but no 5' nucleotidase activity (24). Holtz, et al. (25) isolated and characterized membranes from the cultivated mushroom. Hodges, et al. (26) found an ATPase activity associated with the plasma membrane fraction from oat roots. Leonard, et al. (27) detected six different ATPase activities in an oat root homogenate. Only one of the six activities was associated with the plasma membrane fraction. Patni, et al. (28) isolated plasma membranes from Ochromanas danica, an algae with no cell wall, and reported β-galactosidase, acid phosphatase, alkaline phosphatase, 5' nucleotidase, and (Na^{+}-K^{+} ATPase) activities associated with the plasma membrane. Morre´, et al. (29) found no 5' nucleotidase activity associated with the plasma membrane fraction from onion stems.

CHAPTER III

MATERIALS AND METHODS

A. Plants

Gossypium hirsutum plants were grown in a vermiculite-soil mixture in a Percival controlled environmental growth chamber under 16 hour light periods with Grow-Lux incandescent illumination. Light period temperature was held at 32°C and dark period temperatures were lowered to 21°C. All plants were grown from seeds. Plants from 30-90 days old were used in all experiments.

B. Chemicals and Reagents

All reagents were of analytical grade or of the highest quality available unless otherwise stated.

C. Enzymes and Substrates

Macerase and Cellulysin were purchased from Cal Biochem., La Jolla, Calif. ATP and AMP in the sodium salt form were purchased from Sigma Chemical Co., St. Louis, Mo.

D. Preparation of Protoplasts

Protoplasts were isolated from leaves by a modification of the method of Kassanis, et al. (7). The leaves were surface sterilized for five minutes in 70% ethanol and 0.1% Tween 20 and for twelve minutes in

2.75% sodium hypochlorite and 0.1% Tween 20. The leaves were then rinsed six times with sterile glass distilled water and cut into thin strips. One gram of leaf pieces were then placed in 10 ml of enzyme media that contained: 0.5% macerase, 1.0% cellulysin, 0.35M sorbitol, 0.35M mannitol in 100mM MES buffer pH 5.5. The flask was then covered with aluminum foil and placed in a 30°C temperature room without shaking for 16 hours. The media was filtered through four layers of cheesecloth and centrifuged at 300 x g for five minutes. The pellet was taken up in 0.6 ml of a solution containing 5mM $MgCl_2$, 50mM Tricine buffer pH 8.0 and 0.6M sorbitol. The protoplasts were purified by the method of Kanai and Edwards (30). An aqueous liquid-liquid two phase system consisting of 1.1 ml of 30% (w/w) Polyethylene glycol 6000, 3.0 ml of 20% (w/w) Dextran T_{40}, 0.3 ml of 0.2M Phosphate buffer pH 7.5, and 1.0 ml of 2.4M sorbitol was thoroughly mixed by inversion. The 0.6 ml of protoplast preparation was added to the two phase system and the solution was mixed by inversion and centrifuged at 300 x g for six minutes in a swinging bucket centrifuge. Intact protoplasts were collected at the interface of the two liquid phases while chloroplasts and broken chloroplasts suspended in the lower phase. The intact protoplasts were collected with a disposable pipette.

Protoplasts from suspension cells were isolated by the method of Uchimiya and Murashige (31). The enzyme medium contained 0.4M sorbitol, 0.5% macerase and 0.5% cellulysin dissolved in glass distilled water pH 5.0. Five hundred milligrams of suspension cells were placed in 12.5 ml of enzyme media and shaken at 125 RPM in a 30°C temperature room. After two hours the enzyme medium was filtered through a nylon screen with a 56 micron pore size. Large clumps of cells were retained on the nylon

screen and the free protoplasts passed through the screen.

E. Membrane Isolation

The isolated protoplasts were centrifuged at 100 x g for five minutes and resuspended in 10 mls of 10mM Tris buffer pH 7.4 to lyse the protoplasts. The membranes were then isolated by the method of Patni, et al. (28). The lysed protoplasts were centrifuged at 480 x g for ten minutes. The 480 x g supernatant was then centrifuged at 2,000 x g for twenty minutes. The 2,000 x g supernatant was centrifuged at 15,000 x g for twenty minutes. The 15,000 x g supernatant was then centrifuged for 4 hours at 175,000 x g. The pellet was resuspended in 10mm Tris buffer pH 7.4 which also contained 0.25M sucrose and 1mM EDTA and placed on a 20-45% sucrose gradient. The gradient was centrifuged at 44,000 x g in a SW 25.2 rotor for twelve hours and the fractions assayed for enzymatic activity.

F. Callus Initiation

The cotton callus tissue was initiated by the method of Downing (32). The callus was maintained on SH media as described by Schenk and Hildebrandt (33) and modified by Downing (32).

G. Assays

Protein concentrations were determined by a modification of the Lowry procedure as described by Hartree (34) using bovine serum albumen as a standard. Phosphate was determined by the method of Lazarus, et al. (35). Alkaline Phosphatase assays were performed as described for the E. coli enzyme (36). 5' nucleotidase was assayed by phosphate re-

lease as described by Morre´ (37). ATPase was assayed by a modification of Shin, et al. (38) using 400 mm NaCl, 80 mM KCl, 20 mM $MgCl_2$, 2 mM EDTA and 50 mM ATP. Suspension cells were counted by the method of Uchimiya, et al. (31). A 500 mg sample of suspension cells was added to 5 mls of 5% chromium trioxide and placed on a rotary shaker rotating at 125 RPM. After 24 hours the sample was diluted to 50 ml with glass distilled water. A 10 ml aliquot was placed in a test tube and shaken on a vortex mixer for two minutes. A 10 ul sample was pipetted into the counting chamber and counted. The counting chamber consisted of one sheet of parafilm attached to a microscope slide by spreading vaseline over the slide and sealing the parafilm to the slide by passing the slide through a bunsen burner flame. A hole approximately one centimeter square was cut in the parafilm and the sample placed in the middle of the hole. A coverslip was placed on top of the solution and the sample counted.

Protoplasts were counted by the method of Ruesink (39).

H. Suspension Cells

Between 100 and 500 mgs of callus tissue was placed in 50 mls of SH media without agar and shaken on a gyroshaker at 175 RPM to initiate suspension cell growth. Approximately 100 to 500 mg of cells were subcultured into fresh media at two to three week intervals. After 24 subcultures 1 g of cells was placed in a spinner flask containing 500 ml of SH media. The spinner flask was constructed so that sterile air passed through a small glass tube which was inserted through a rubber stopper in the side-arm of the flask. The other side-arm of the spinner flask contained a similar glass tube except that this glass tube extended down

into the media. A 10 ml sample was removed every two days by using air pressure from an air pump through one side-arm of the flask. The cells and media were collected at the other side-arm in a graduated test tube. The cells were collected on miracloth. The miracloth was weighed before and two days after the filtering process to determine the dry weight of cells per 10 ml sample.

I. Membrane Isolation Using Pulverized Leaves

Leaves were ground in a mortar and pestle with buffer which contained 10 mM Tris pH 7.2, 3 mM EDTA, and 0.25 M sucrose. Acid washed sea sand and 0.1 grams of Polyclar AT per gram of leaf tissue were also in the grinding media. The leaves were ground for ten minutes in a $4^{o}C$ cold room. The suspension was then filtered through four layers of cheesecloth. The mortar and pestle were then rinsed with grinding buffer and the combined filtrates were centrifuged at 100 x g for five minutes. The supernatant was centrifuged at 15,000 x g for twenty minutes. The 15,000 x g supernatant was centrifuged at 105,000 x g for two hours and the pellet was resuspended in 18% sucrose. The 18% sucrose was layered on a 20 to 45% discontinuous sucrose gradient and centrifuged at 44,000 x g in an SW 25.2 rotor. Six ten ml fractions were collected from the gradient. The fractions were centrifuged at 105,000 x g for seventy minutes and the pellets were taken up in 2 ml of buffer containing 10 mM Tris pH 7.2, 1 mM EDTA 1 mM $CaCl_2$ and 1 mM $MgSO_4$.

CHAPTER IV

RESULTS AND DISCUSSION

A. Callus Tissue

Callus tissue was initiated and maintained on SH media. Table I shows the composition of SH media. A growth curve for cotton callus tissue is shown in Figure 1. This figure shows that cotton callus grows logarithmically for the first three weeks and then begins to level off. The callus began to turn green after six weeks and started turning brown after seven weeks. It was decided from the growth curve data and from visual observations to subculture the callus every four weeks.

Attempts were made to initiate roots and shoots from the callus by varying the auxin cytokinin ratio. The normal auxin cytokinin ratio is 25:1. The auxin cytokinin ratio was varied from 50 to 150:1 for root initiation and from 2.5 to 12.5:1 for shoot initiation. In these experiments α Naphthalene Acetic Acid was used as the auxin, but no roots or shoots were observed.

B. Suspension Cells

The growth curve for cells in suspension is shown in Figure 2. This figure indicates suspension cells reach their maximum growth in three weeks. Suspension cells were subcultured every two to three weeks. Figure 3 shows the same data plotted in Figure 2 plotted as

TABLE I

SH MEDIA COMPOSITION

Major Elements	mg/l For 4 X Solution	Minor Elements	mg/l For 100 X Solution
KNO_3	10,000	$MnSO_4 \cdot H_2O$	1,000
$MgSO_4 \cdot 7H_2O$	1,600	H_3BO_3	500
$NH_4H_2PO_4$	1,200	$ZnSO_4 \cdot 7H_2O$	100
$CaCl_2$	604	KI	100
		$CuSO_4 \cdot 5H_2O$	20
		$Na_2MoO_4 \cdot 2H_2O$	10
		$CoCl_2 \cdot 6H_2O$	10

Iron

1.5 grams $FeSO_4 \cdot 7H_2O$ and 2.0 grams Na_2EDTA in 100 ml H_2O and heated to 80°C for a 1000 X solution.

Growth Regulating Substances

2,4-dichlorophenoxy-acetic acid	50)	
p-chlorophenoxy-acetic acid	200)	mg/l in a 100 X Solution
6-furfurlyaminopurine	10)	

Vitamins

Thiamine HCl	500)	
Nicotinic Acid	500)	mg/100 ml in 1000 X Solution
Pyridoxine HCl	500)	

Inositol 1 gram/l

Sucrose 30 grams/l

pH adjusted to 5.6 - 5.8

Figure 1. Growth Curve of Gossypium hirsutum Callus Tissue on SH Media (See Text for Experimental Details)

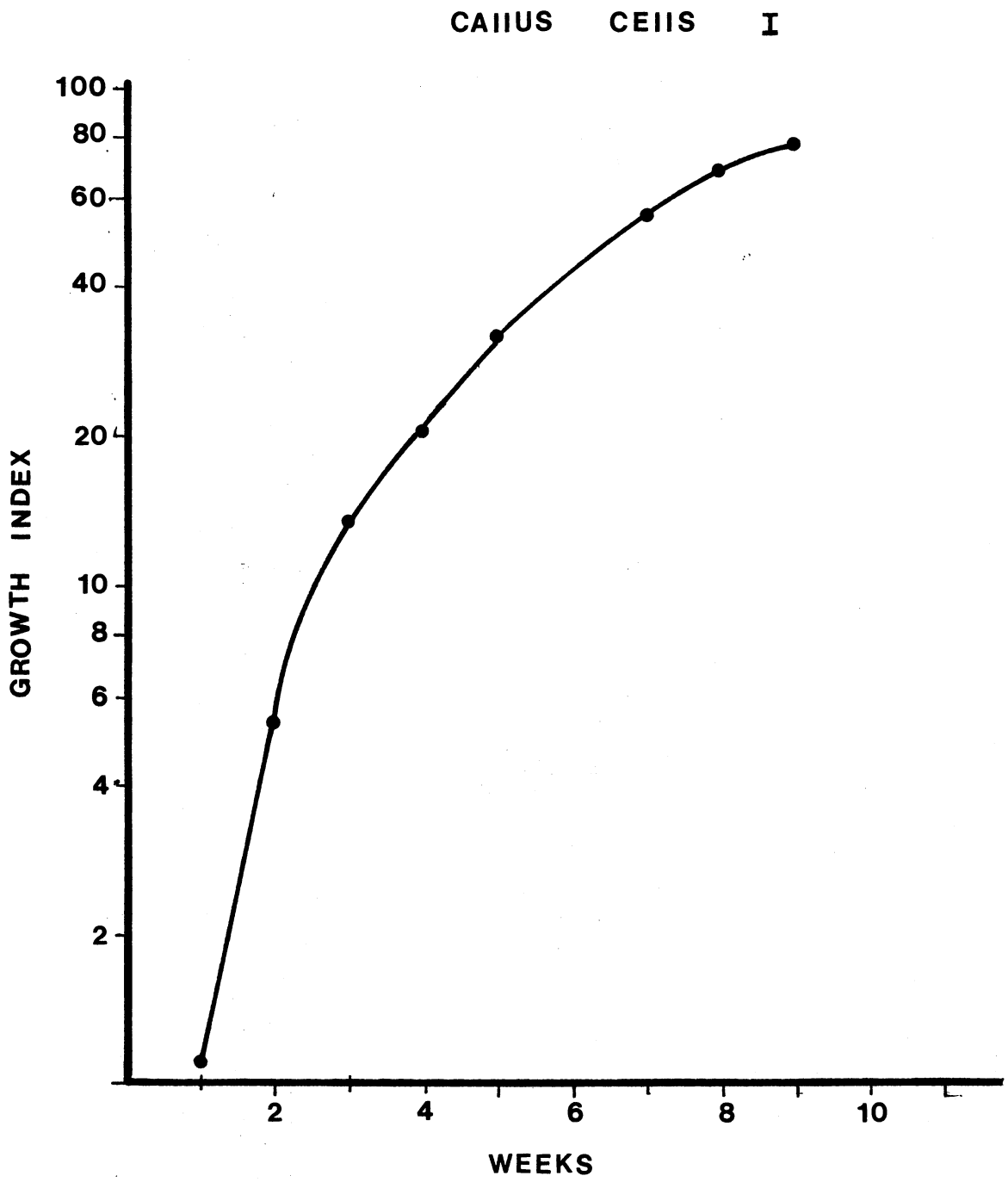

CAIIUS CEIIS I
100
80
60
40
20
10
8
6
4
2
GROWTH INDEX
2
4
6
8
10
WEEKS

Figure 2. Growth Curve of Gossypium hirsutum Suspension Cells in SH Media (See Text for Experimental Details)

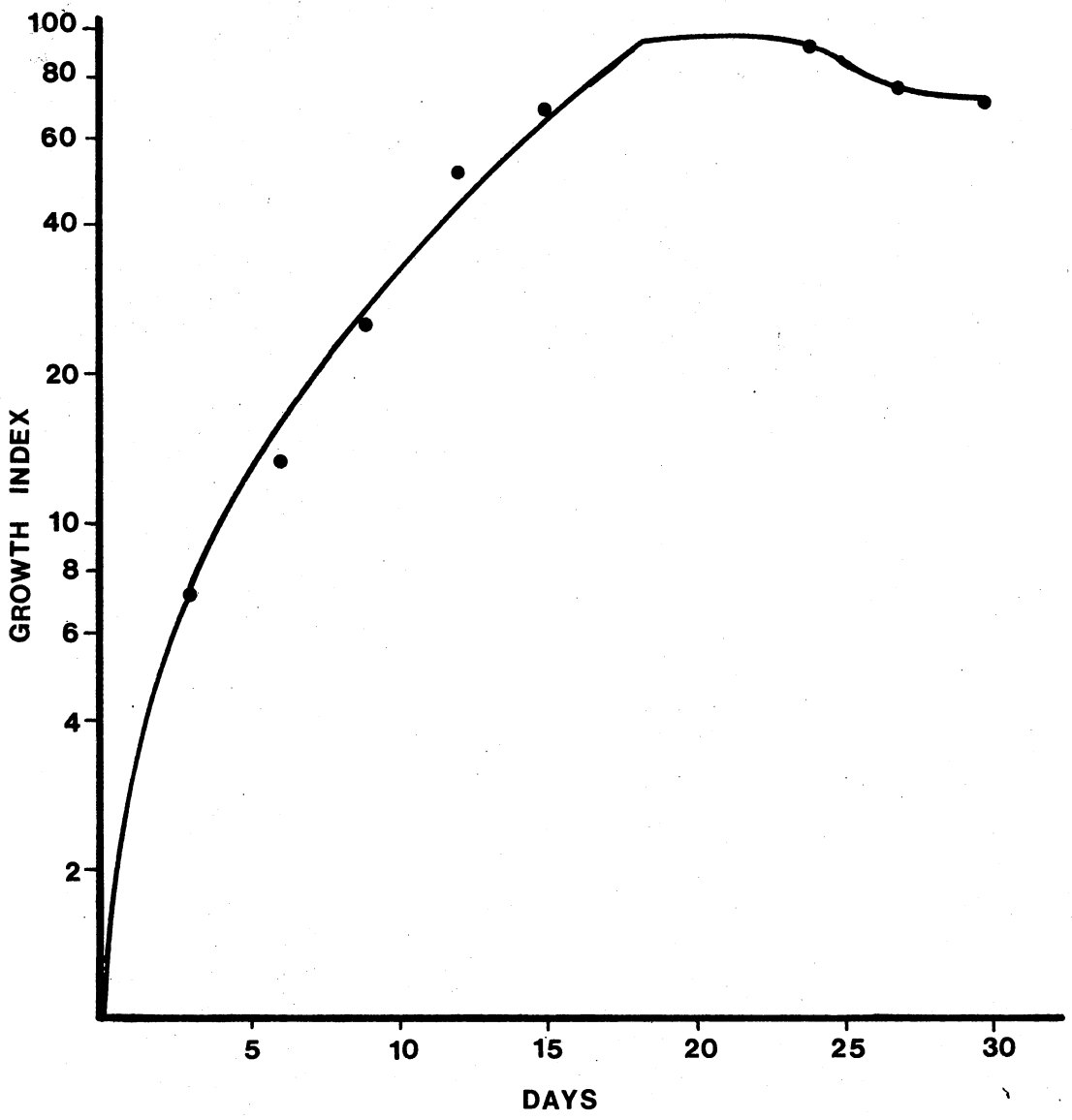
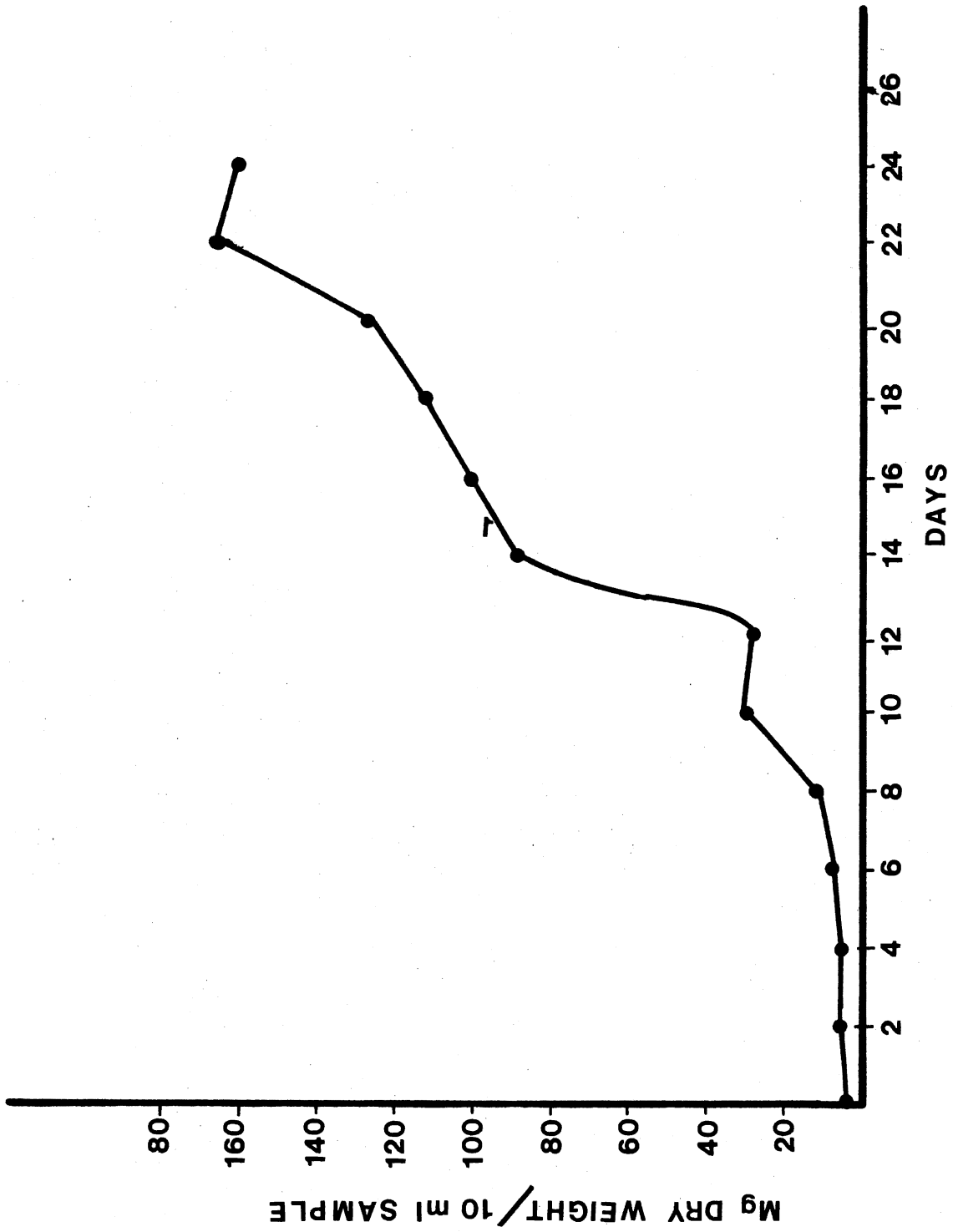

SUSPENSION CELLS II
GROWTH INDEX
100
80
60
40
20
10
8
6
4
2
5
10
15
20
25
30
DAYS

Figure 3. Increase in Dry Weight of Cells/10 ml Sample With Time (See Text for Experimental Details)


Mg DRY WEIGHT/10 ml SAMPLE
20
40
60
80
100
120
140
160
180
2
4
6
8
10
12
14
16
18
20
22
24
26
DAYS
7

mg dry weight of cells/10 ml aliquot vs. time. This graph also shows the maximum amount of growth was achieved after three weeks. A plot of pH vs. time is shown for cells in suspension in Figure 4. When the pH profile (Figure 4) is compared to the growth index (Figure 3) it can be seen that the pH drops as the cells are in lag phase, increases when the cells are in log phase and levels off near the original pH when the cells reach stationary phase.

C. Suspension Protoplasts

Protoplasts from suspension cells were initially isolated using 1% cellulysin, 0.5% macerase 0.7M sorbitol and 100 mM MES buffer pH 5.5. Since the yield of protoplasts was so low it was decided to optimize the conditions for their isolation. All cells used in the optimization experiments were 14 day old Ac-44 leaf cells. The order of the appearance of the figures indicates the order of the experiments. Conditions optimized in the first experiments were then used in later experiments. Figure 5 shows the relationship between the percent protoplasts formed and the concentration of cellulysin. A concentration of 0.5% cellulysin gave the best results. Since the first concentration of cellulysin used gave the best results, an experiment with 0.25% cellulysin on other cells was conducted to determine if a concentration less than 0.5% cellulysin gave better results, however, the results of this experiment confirmed that 0.5% cellulysin produced maximal protoplasts.

A plot of the percent protoplasts and the concentration of macerase is shown in Figure 6. A concentration of 0.5% macerase presented the highest yield of protoplasts.

The relationship between the percent protoplast and pH is illus-

Figure 4. Variation of pH of Suspension Cells During Growth (See Text for Experimental Details)

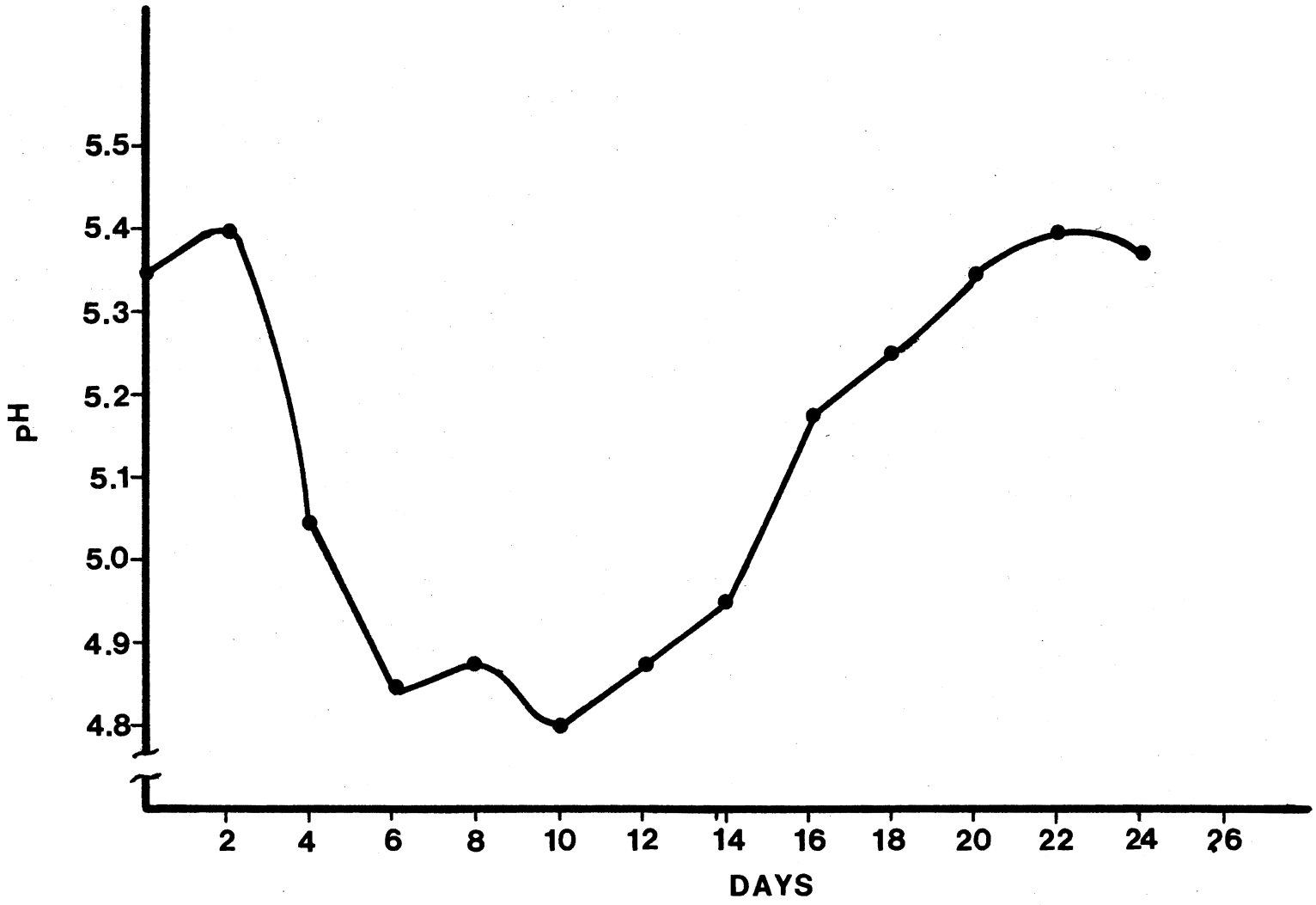

pH
5.5
5.4
5.3
5.2
5.1
5.0
4.9
4.8
2
4
6
8
10
12
14
16
18
20
22
24
26
DAYS

Figure 5. Yield of Protoplasts From Suspension Cells With Varying Concentrations of Cellulysin (See Text for Experimental Details)

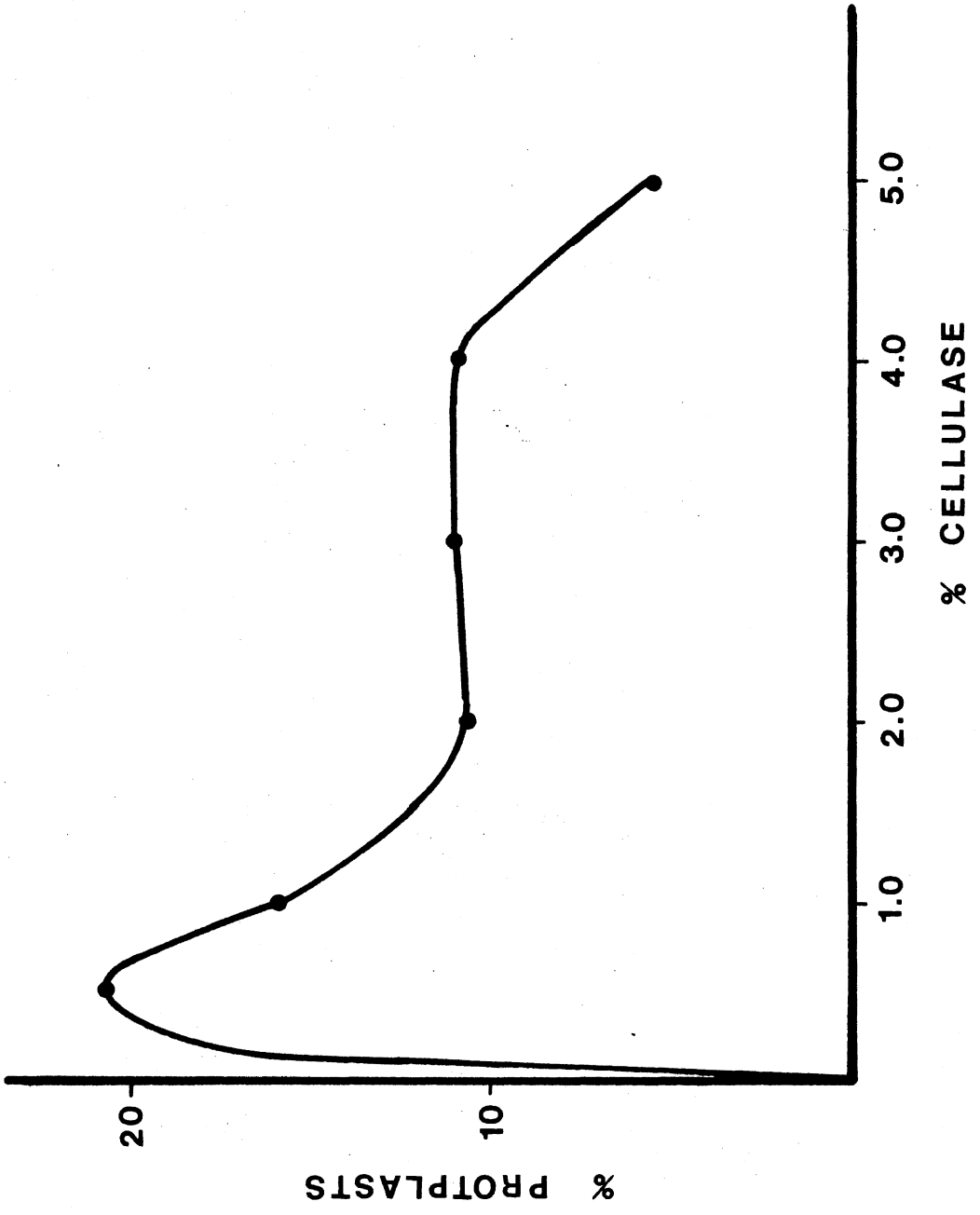
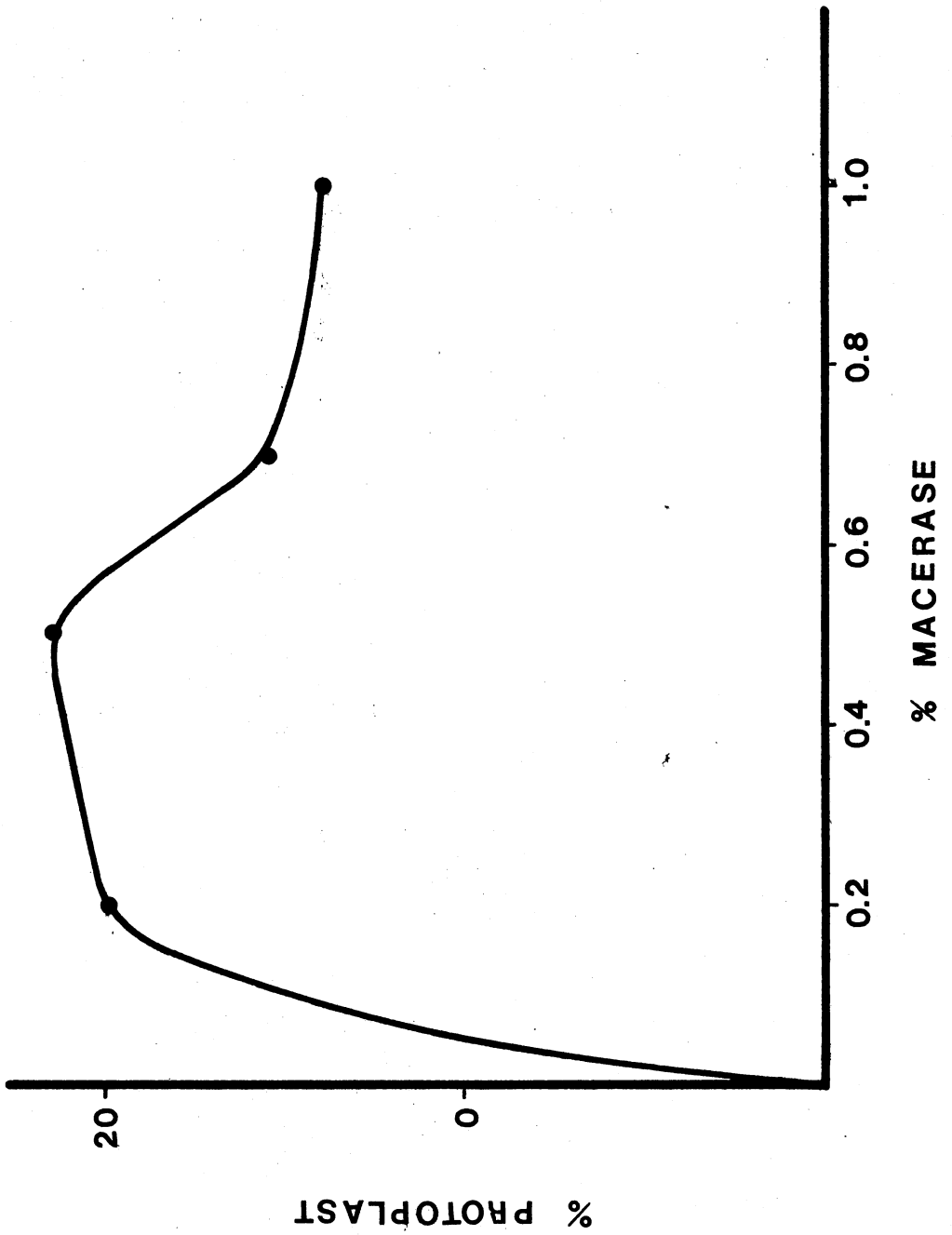

% PROTPLASTS
20
10
1.0
2.0
3.0
4.0
5.0
% CELLULASE

Figure 6. Yield of Protoplasts From Suspension Cells With Varying Concentrations of Macerase (See Text for Experimental Details)


% PROTOPLAST
20
0
0.2
0.4
0.6
0.8
1.0
% MACERASE

trated in Figure 7. At pH 5.0 the best results were consistently obtained although pH 6.5 gave similar yields of protoplasts.

Figure 8 shows the relationship between the percent protoplasts formed and the concentration of sorbitol. At a concentration of 0.2 M sorbitol and below the protoplasts are not stable. The percent protoplasts formed increases up to 0.4 M sorbitol and then decreases at concentrations above 0.4 M sorbitol.

The release of protoplasts with time is plotted in Figure 9. Two hours is the optimum time for isolation with the yield at three hours slightly less. Subsequent protoplast isolation experiments were conducted between two and three hours.

Figure 10 shows that a volume of 12.5 mls of enzyme solution per 500 mg wet weight of cells gave the highest yield of protoplasts.

The effect of shaking speed is shown in Figure 11. A rate of 175 RPM gave the best results. Later experiments with two and four day old cells indicate that 175 RPM is too fast a shaking speed and results in the breakage of many protoplasts. This may indicate that older cells have membranes which are stabilized by a factor or factors which younger cells do not have.

Figure 12 indicates that cells in the middle of logarithmic growth gave the highest yields of protoplasts. Protoplasts from suspension cells are normally isolated in the light with shaking.

D. Leaf Protoplasts

A comparison of different sterilization procedures for the isolation of protoplasts from leaf tissue is shown in Table II. Five minutes in 70% ethanol and ten minutes in 2.75% sodium hypochlorite gave similar

Figure 7. Yield of Protoplasts From Suspension Cells Varying pH (See Text for Experimental Details)

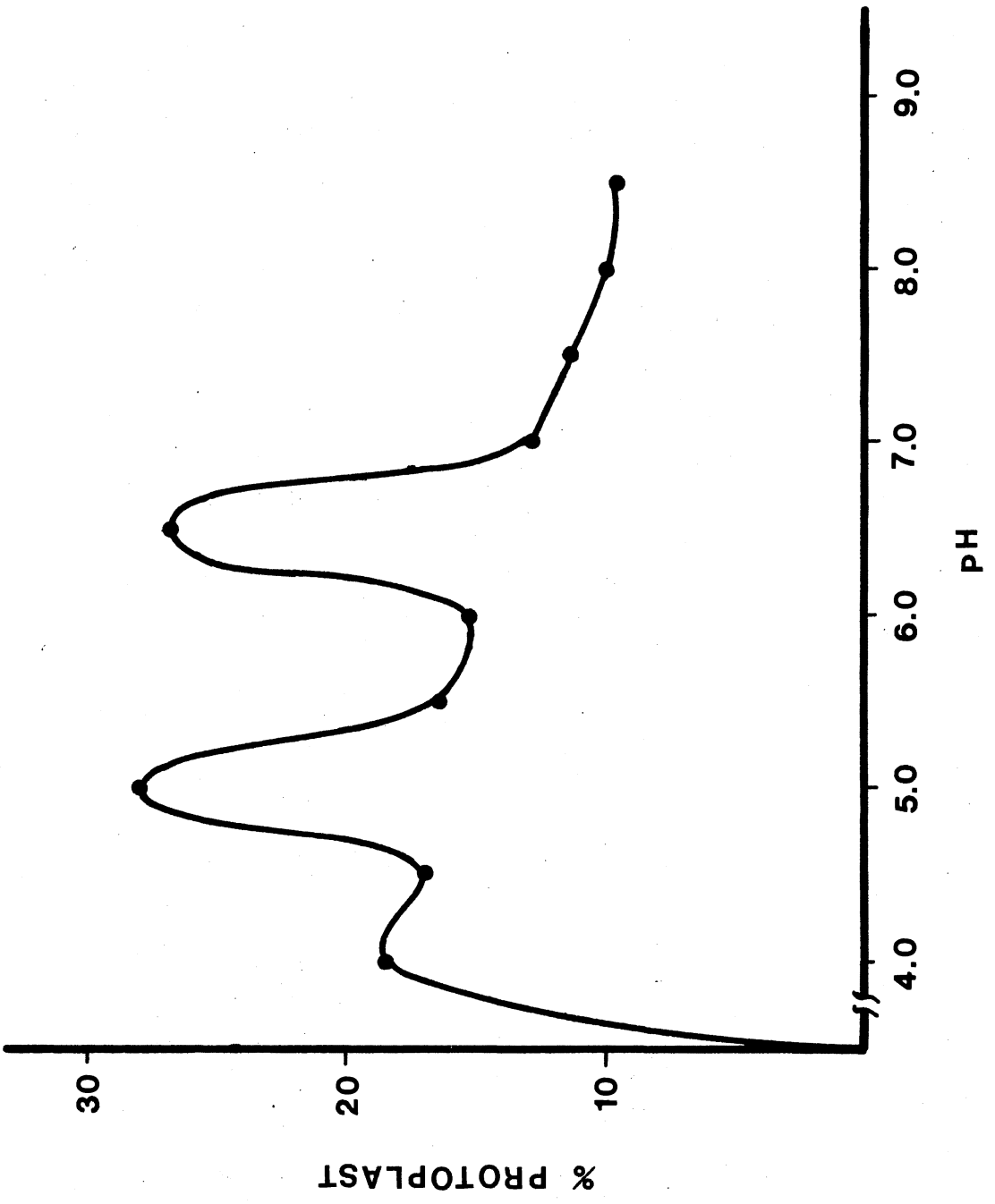

% PROTOPLAST
30
20
10
4.0
5.0
6.0
7.0
8.0
9.0
pH

Figure 8. Yield of Protoplasts From Suspension Cells With Varying Concentrations of Sorbitol (See Text for Experimental Details)

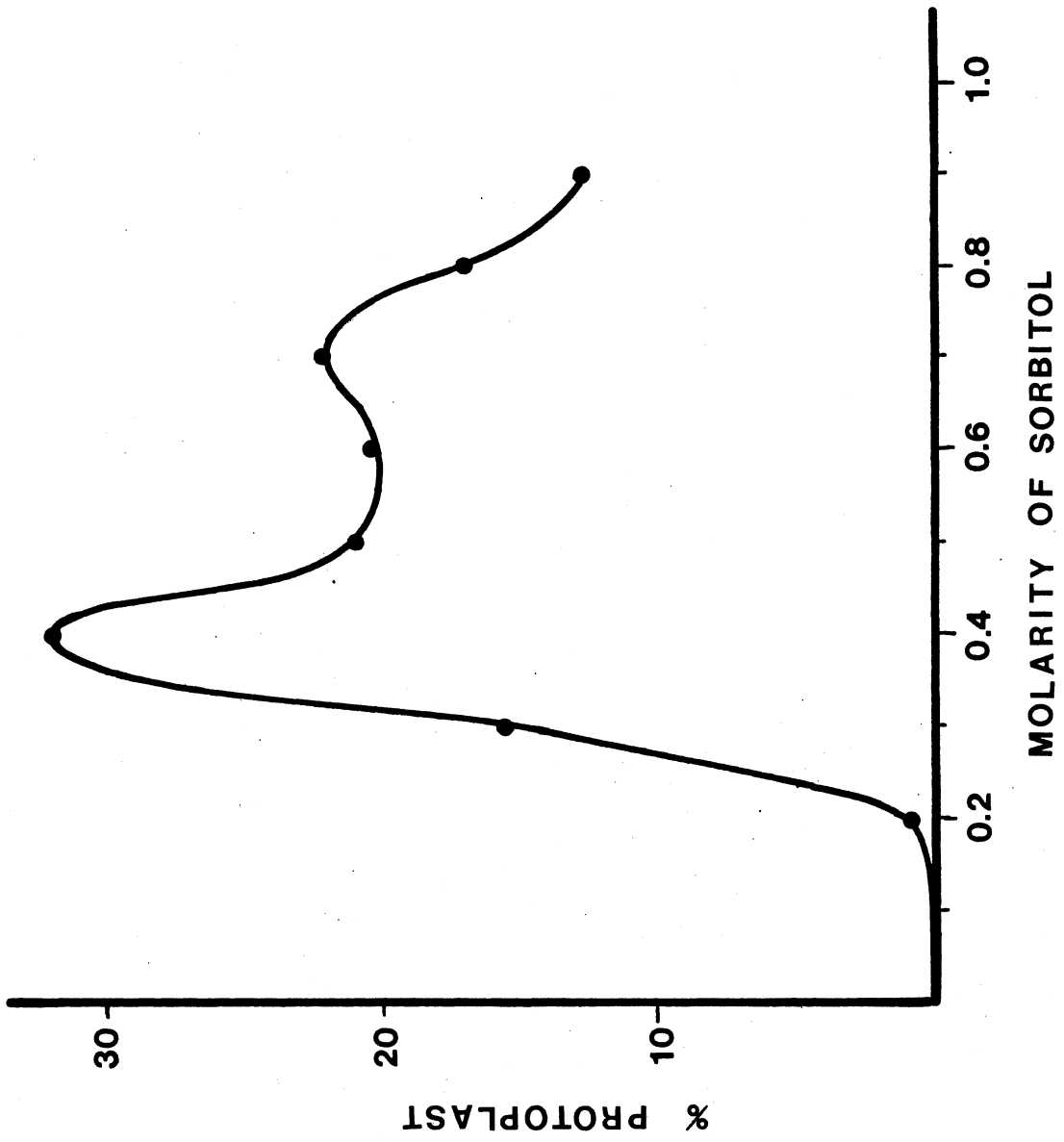

% PROTOPLAST
10
20
30
0.2
0.4
0.6
0.8
1.0
MOLARITY OF SORBITOL

Figure 9. Yield of Protoplasts From Suspension Cells With Time (See Text for Experimental Details)

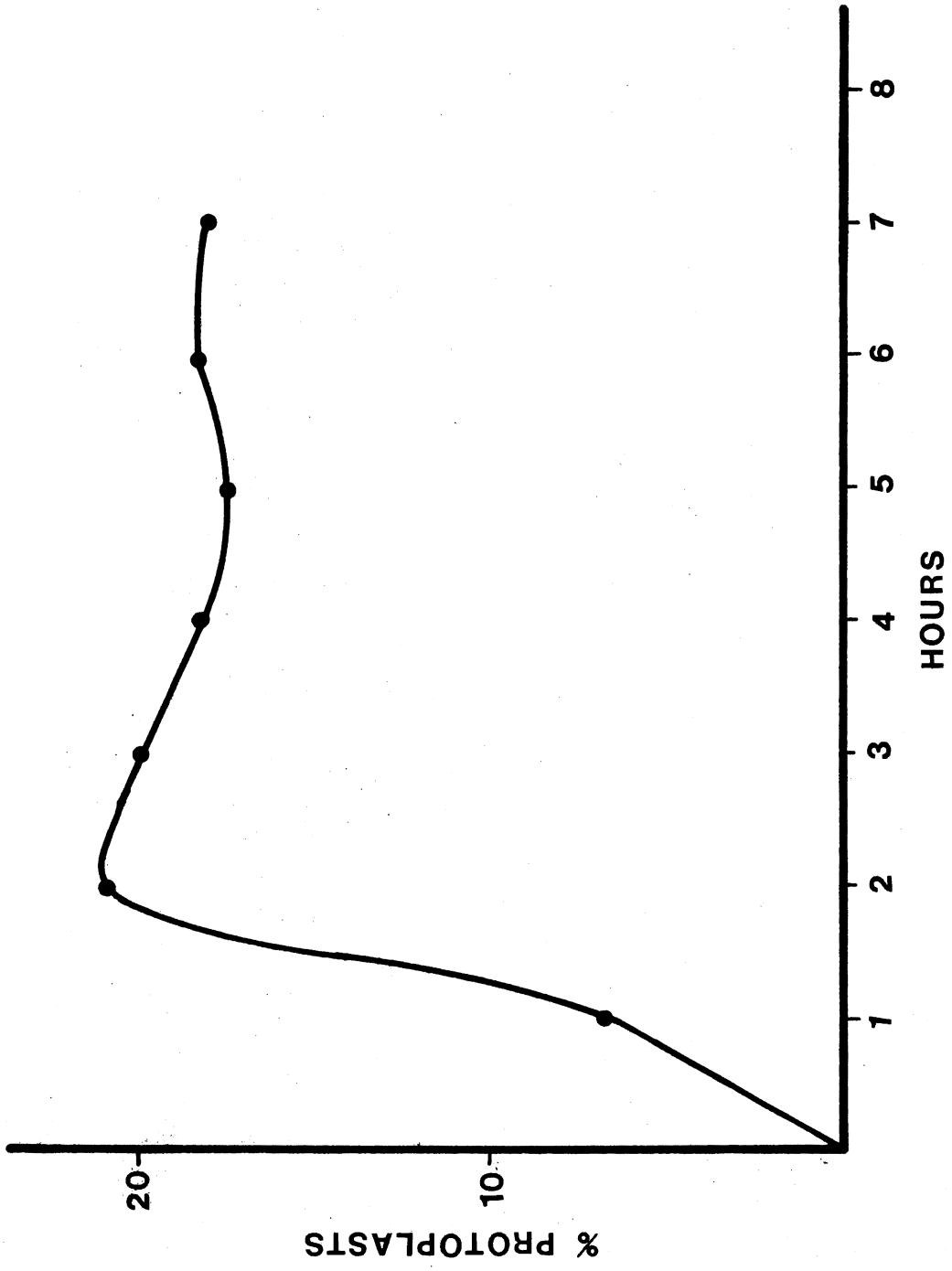

% PROTOPLASTS
20
10
HOURS
1
2
3
4
5
6
7
8

Figure 10. Yield of Protoplasts From Suspension Cells Varying the Volume of Enzyme Media/500 mg of Cells (See Text for Experimental Details)

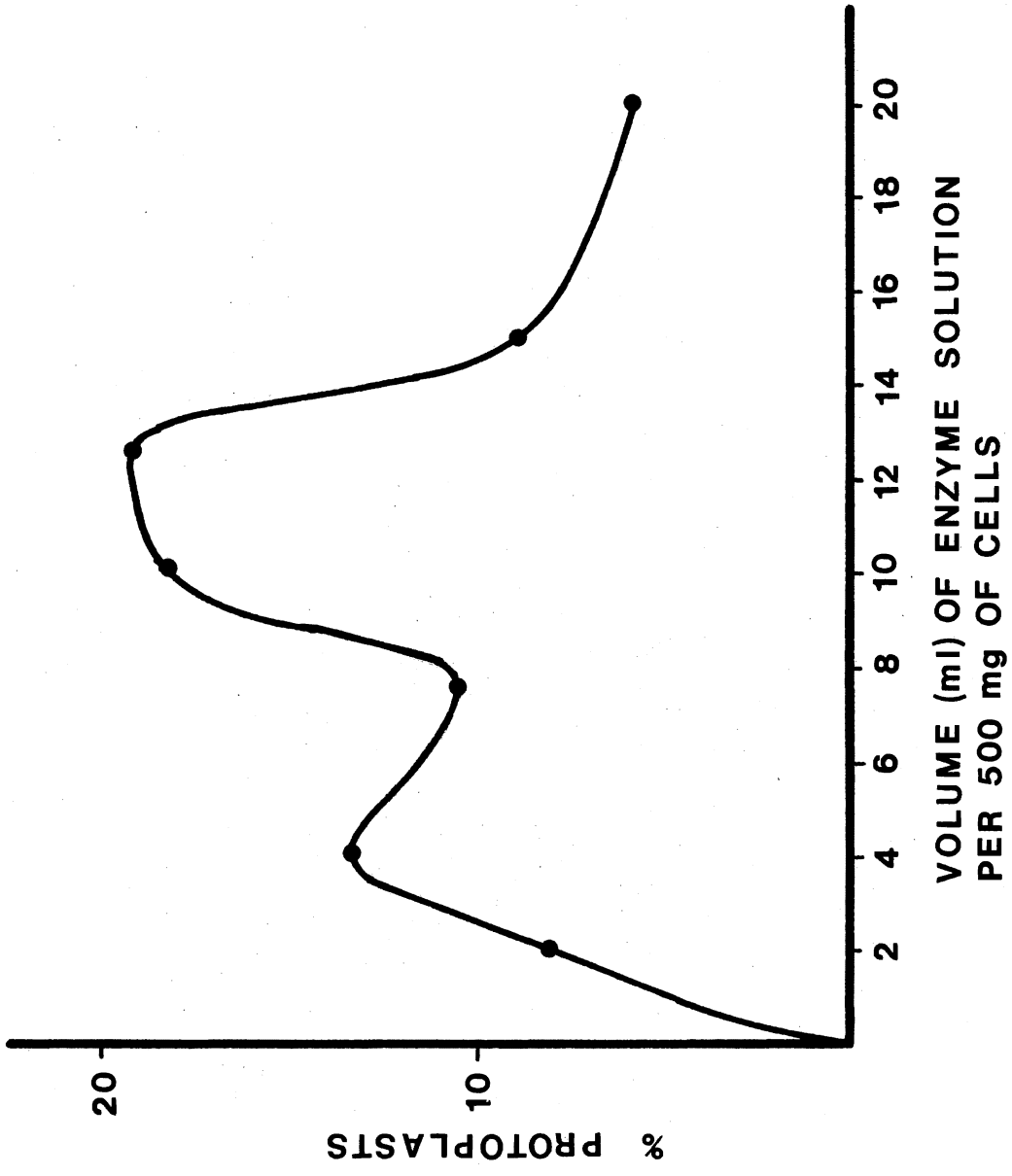

% PROTOPLASTS
20
10
2
4
6
8
10
12
14
16
18
20
VOLUME (ml) OF ENZYME SOLUTION
PER 500 mg OF CELLS

Figure 11. Yield of Protoplasts From Suspension Cells Varying the Shaking Speed (See Text for Experimental Details)

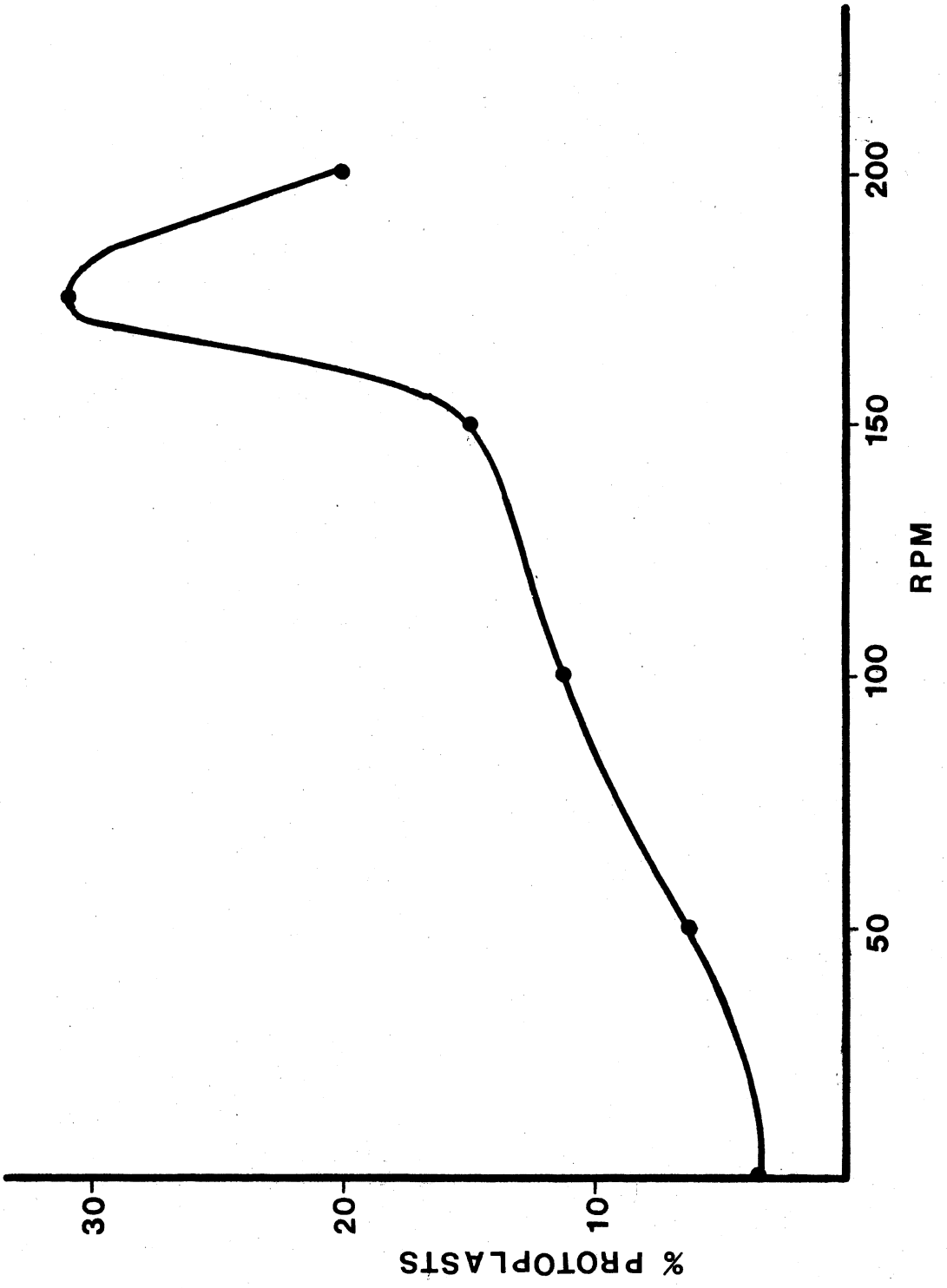

% PROTOPLASTS
10
20
30
RPM
50
100
150
200

Figure 12. Yield of Protoplasts From Suspension Cells During Different Stages of Growth (See Text for Experimental Details)

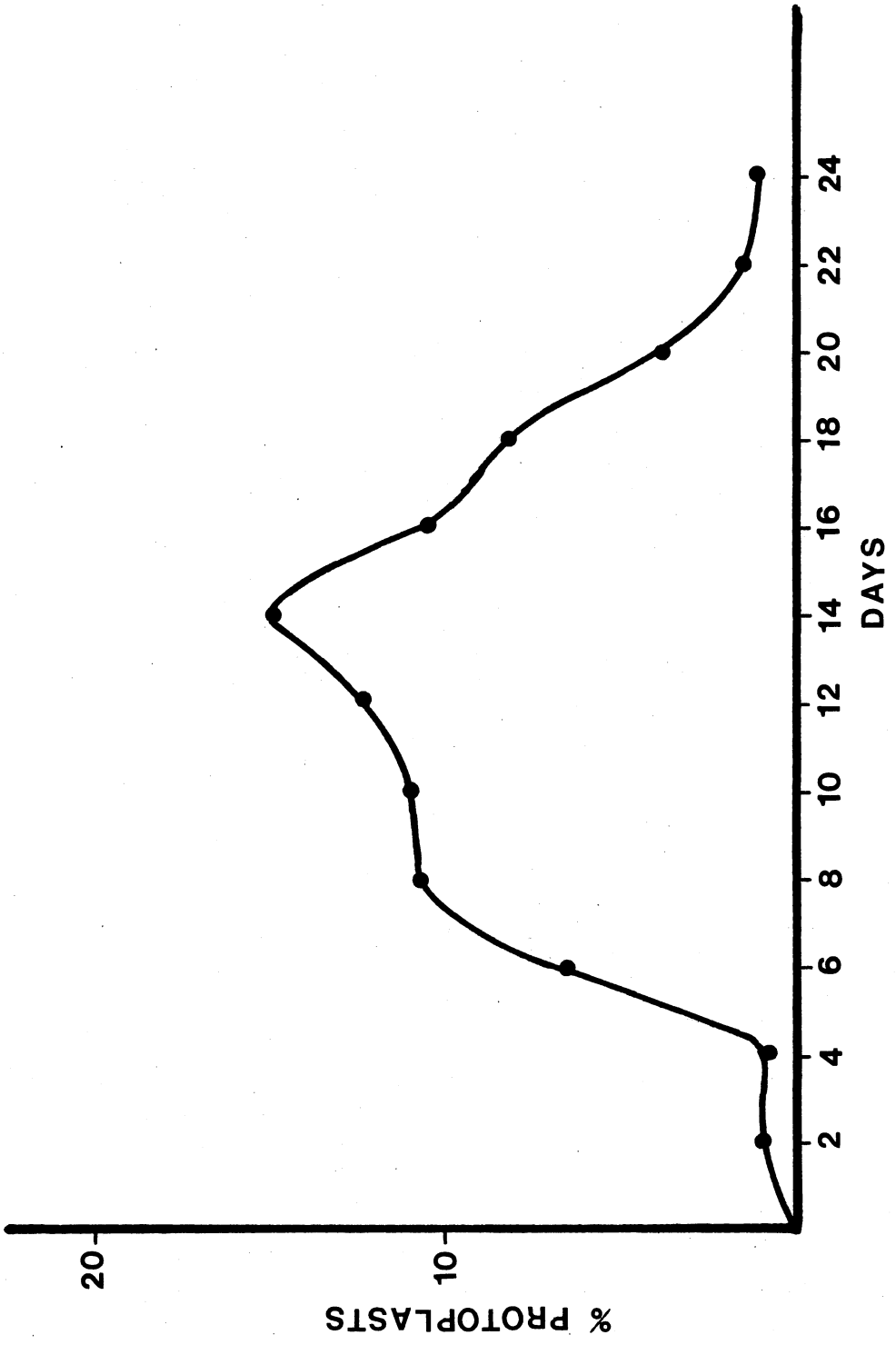

% PROTOPLASTS
20
10
2
4
6
8
10
12
14
16
18
20
22
24
DAYS

TABLE II

EFFECT OF TIME IN STERILIZATION WASH ON CONTAMINATION AND PROTOPLAST FORMATION

Ethanol (Min.)	NaOCl	Contamination	Protoplast Formation
5	10	0	2
10	5	1	2
2	12	2	0
4	12	0	1
2	15	1	1

0 = No contamination or protoplast formation.

1 = Small amounts of contamination or protoplasts.

2 = Large amounts of contamination or protoplasts.

results to four minutes in ethanol and twelve minutes in sodium hypochlorite. The five and ten minute procedure was used since it gave a higher yield of protoplasts and subjected the leaf to less time in hypochlorite solution.

Table III shows that both light and shaking cannot be used in the isolation of protoplasts from leaf tissue. Viable protoplasts were found only in the flask which was kept in the dark and not shaken. Photosynthetic products of the leaf chloroplasts may be released, and these products may cause the protoplasts to rupture. As is the case with suspension cells, young leaf tissue gives a better yield of protoplasts than older leaf tissue.

Figure 13 shows a flow chart of the procedure for the isolation of membranes. The pellet from the 175,00 x g centrifugation was resuspended in a small volume of Tris buffer pH 7.4 which also contained 0.25 M sucrose and 1 mm EDTA and placed on a 20-70% sucrose gradient. The material containing the 5' nucleotidase activity was always in the pellet. This activity may have been due to phosphatase activity, but the phosphatase activity was never checked due to a lack of material. Experiments with alkaline phosphatase enzyme purchased from Sigma Chemical Co. showed that alkaline phosphatase can hydrolyze 5' AMP.

E. Isolating Membranes From Leaves by Grinding in a Mortar and Pestle

Membranes can also be isolated from leaves by grinding in a mortar and pestle. A major advantage of the grinding method is that large amounts of material can be used. The major disadvantage of the grinding method is that one could get contamination from cell organelle membranes

TABLE III

THE EFFECT OF LIGHT AND SHAKING ON THE ISOLATION OF PROTOPLASTS FROM COTTON LEAF CELLS

Conditions for Isolation	Protoplasts Formed
In light with shaking	-
In light without shaking	-
In darkness with shaking	-
In darkness without shaking	+

- = No protoplasts were formed.

+ = Protoplasts were formed.

Figure 13. Flow Chart for the Preparation of Membranes From Protoplasts (See Text for Experimental Details)

Lysed Protoplasts - 480 x g - 10 min.

Pellet	Supernatant
Nuclei, Cell Debris Whole Cells	2000 x g 20 minutes

Pellet	Supernatant
Chloroplasts	15,000 x g 20 minutes

Pellet	Supernatant
Mitochondria	175,00 x g 4 hours
	Membranes and E. R. Ribosomes

and when one grinds cells chloroplast and mitochondrial membranes are ruptured these fragments may centrifuge down with the plasma membranes. This contamination could complicate the membrane isolation since a lack of good "marker enzymes" is one of the problems in isolating plant plasma membranes. The largest amount of 5' nucleotidase activity was found in the 25 and 30% sucrose fractions from a density gradient centrifugation. The activity detected, 10-20 umoles/hr/mg protein, is low compared with the values reported in the literature (28).

CHAPTER V

CONCLUSIONS

Cotton callus and suspension cells have been initiated and maintained. Protoplasts were isolated from both leaf and suspension cells. A procedure for the isolation of membranes has been worked out.

The main problem in isolating plant membranes appears to be the lack of a good "marker enzyme" for the plant plasma membrane. A review of the literature indicates that Na^+-K^+ ATPase is a good "marker enzyme" for root tissue. However, to this time no reliable "marker enzyme" has been found for the plasma membrane of leaf tissue.

Another problem is the low yield of protoplasts from both leaf and suspension cells. Since it cannot be determined how many cells are in a leaf, it was not possible to calculate a percent yield of protoplasts from leaf tissue. It doesn't appear, however, that the yield of protoplasts from leaf cells is as great as the yield from suspension cells. Another problem is that the yield of protoplasts from suspension cells is greatest at a point on the growth curve where the cells have only reached one half their maximum growth.

Studies are in progress to determine if the lysed protoplasts can be frozen and stored until a large enough quantity of material is available. A good "marker enzyme" is also being sought.

SELECTED BIBLIOGRAPHY

1. Klercker, J. von (1892) Öfvers. Kungl Vetensk. Fork, Stockh., 49, 463-465.

2. Vreugdenker, D. (1957) Acta Bot. Neer., 6, 472.

3. Cocking, E. C. (1960) Nature, 187, 962.

4. Gregory, D. W. and Cocking, E. C. (1963) Biochem. J., 88, 40P.

5. Takebe, I., Otsuki, Y. and Aoki, A. (1968) Plant Cell Physiol., 9, 115-124.

6. Power, J. B. and Cocking, E. C. (1969) Biochem. J. 111, 33P.

7. Kassanis, B. and White, R. F. (1974) J. Gen. Virology, 24, 447-452.

8. Carlson, P. S., Smith, H. H. and Dearing, D. D., Proc. Natl. Acad. Sci. U. S., 69, 2292-2294.

9. Kao, K. N., Constabel, F., Michayluk, M. R. and Gamborg, O. L. (1974) Planta, 120, 215-227.

10. Moore, T. S. and Beevers, H. (1974) Plant Physiol., 53, 261-265.

11. App, A. and Granados, R. R. (1974) Planta, 120, 201-205.

12. Schenk, R. U. and Hildebrandt, A. C. (1969) Crop Science, 9, 629-631.

13. Davis, D. G., Dusabek, K. E. and Hoerauf, R. A. (1974) In Vitro, 9, 395-398.

14. White, P. R. and Grove, A. R., Eds. (1965) "International Conference on Plant Tissue Culture," McCutchan Publishing Corp., Berkeley.

15. Kruse, Jr., P. F. and Patterson, Jr., M. K., Eds. (1973), "Tissue Culture, Methods and Application," Academic Press, New York.

16. Street, H. E. and Henshaw, G. G. (1966) in "Cells and Tissues in Culture" (Willmer, E. N., Ed.), Vol. 3, pp. 459-533, Academic Press, New York.

17. Street, H. E. (1966) in "Cells and Tissues in Culture" (Willmer, E. N., Ed.), Vol. 3, pp. 534-630, Academic Press, New York.

18. Street, H. E. (1966) in "Cells and Tissues in Culture" (Willmer, E. N., Ed.), Vol. 3, pp. 631-690, Academic Press, New York.

19. Hall, J. L. (1969) Planta, 85, 105-107.

20. Hall, J. L. and Butt, V. S. (1969) J. Exp. Bot., 20, 751-762.

21. Schultz, T. M. and Thompson, J. E. (1969) Biochem. Biophys. Acta, 193, 203-211.

22. Lai, Y. F. and Thompson, J. E. (1970) Phytochemistry, 9, 1017-1021.

23. Lai, Y. F., Thompson, J. E. and Barrel, R. W. (1971) Phytochemistry, 10, 41-49.

24. Lai, Y. F. and Thompson, J. E. (1971) Biochem. Biophys. Acta, 233, 84-90.

25. Holtz, R. B., Stewart, P. S. and Patton, S. (1972) Plant Physiol., 50, 541-546.

26. Hodges, T. K., Leonard, R. T., Bracker, C. E. and Keenan, T. W. (1972) Proc. Natl. Acad. Sci. U. S., 69, 3307-3311.

27. Leonard, R. T., Hansen, D. and Hodges, T. K. (1973) Plant Physiol., 51, 749-754.

28. Patni, N. J., Billmire, E. and Aaronson, S. (1974) Biochem. Biophys. Acta, 373, 347-355.

29. Morre, D. J., Roland, J. C. and Lembi, C. A. (1970) Proc. Ind. Acad. Sci., 79, 96-106.

30. Kanai, R. and Edwards, G. E. (1973) Plant Physiol., 52, 484-490.

31. Uchimiya, H. and Murashige, T. (1974) Plant Physiol., 54, 936-944.

32. Downing, M. R., "Mevalonate Metabolism in Cell-Free Systems From N. cataria (Catnip) Leaf and Static Cytoculture Tissue" (unpub. Ph.D. dissertation, Oklahoma State University, 1974).

33. Schenk, R. V. and Hildebrandt, A. C. (1972) Can. J. Bot., 50, 199-204.

34. Hartree, E. F. (1972) Anal. Biochem., 48, 422-427.

35. Lazarus, L. H. and Chou, S. C. (1972) Anal. Biochem., 45, 557-566.

36. "Worthington Enzyme Manual" (1972) Worthington Biochemical Corp., Freehold, New Jersey, pp. 73-74.

37. Morre, D. J. (1971) in "Methods in Enzymology", Vol. 22, pp. 137-138.

38. Shin, B. C. and Carraway, K. L. (1973) Biochem. Biophys. Acta, 330, 254-268.

39. Ruesink, A. W. (1971) in "Methods in Enzymology", Vol. 23A, pp. 197-207.

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